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CENP-A Phosphorylation by Aurora-A in Prophase Is Required for Enrichment of Aurora-B at Inner Centromeres and for Kinetochore Function

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Summary

The Aurora (Ipl1)-related kinases are universal regulators of mitosis. We now show that Aurora-A, in addition to Aurora-B, regulates kinetochore function in human cells. A two-hybrid screen identified the kinetochore component CENP-A as a protein that interacts with Aurora-A. Aurora-A phosphorylated CENP-A in vitro on Ser-7, a residue also known to be targeted by Aurora-B. Depletion of Aurora-A or Aurora-B by RNA interference revealed that CENP-A is initially phosphorylated in prophase in a manner dependent on Aurora-A, and that this reaction appears to be required for the subsequent Aurora-B-dependent phosphorylation of CENP-A as well as for the restriction of Aurora-B to the inner centromere in prometaphase. Prevention of CENP-A phosphorylation also led to chromosome misalignment during mitosis as a result of a defect in kinetochore attachment to microtubules. Our observations suggest that phosphorylation of CENP-A on Ser-7 by Aurora-A in prophase is essential for kinetochore function.

Introduction

Chromosome segregation in mitosis requires prior alignment of the complete set of chromosomes at the cell equator. Kinetochores, which assemble at the primary chromosome constriction and comprise centromeric chromatin and multiple protein complexes, play an important role in chromosome alignment. They provide a site for the attachment of spindle microtubules that allows generation of the force required for chromosome movement. Unattached or tension-free kinetochores generate signals that activate the spindle assembly checkpoint and thereby prevent chromosome segregation until all chromosomes are aligned (Rieder and Salmon, 1998; Shah and Cleveland, 2000). The mammalian kinetochore consists of two layers discernible by electron microscopy: the inner plate and the outer domain (Rieder and Salmon, 1998). Proteins that reside in the inner plate constitutively associate with the centromeric chromatin (Earnshaw and Rothfield, 1985). In contrast, the outer domain of the kinetochore is formed as a result of the recruitment of several microtubule binding motor proteins and cell cycle regulatory proteins (Rieder and Salmon, 1998; Shah and Cleveland, 2000; Hoyt, 2001). The mechanisms by which the assembly of kinetochore proteins is regulated and by which these proteins interact with centromeric chromatin to build a functional kinetochore remain unclear, however.

CENP-A is a well-conserved variant of histone H3 and substitutes for this protein in the nucleosome core of centromeric chromatin at the inner plate of the kinetochore (Sullivan et al., 1994; Yoda et al., 2000). CENP-A (or its homolog, Cse4) is implicated in kinetochore assembly in a variety of organisms including yeast, nematodes, flies, mouse, and human. In the absence of CENP-A, kinetochores fail to recruit several components important for their function (Howman et al., 2000; Blower and Karpen, 2001; Oegema et al., 2001; Goshima et al., 2003). Depletion of CENP-A by RNA interference (RNAi) in HeLa cells resulted in mistargeting of the kinetochore proteins CENP-C, CENP-I (hMis6), and CENP-H as well as the failure of chromosomes to align at the metaphase plate (Goshima et al., 2003). On the basis of these observations, CENP-A is thought to be crucial for kinetochore assembly and function (Cleveland et al., 2003).

A screen for mutants with an increased ploidy in budding yeast resulted in the identification of the kinase Ipl1 (Chan and Botstein, 1993). The unstable ploidy in Ipl1 mutants was subsequently shown to result from deficient kinetochore function (Biggins et al., 1999), and Ipl1 was further found to reverse improper kinetochore-microtubule attachments (Tanaka et al., 2002). Aurora kinases are the metazoan counterparts of yeast Ipl1 and play important roles in various mitotic events (Bischoff and Plowman, 1999; Nigg, 2001). Three mammalian isoforms of Aurora have been identified to date, called Aurora-A, -B, and -C (Nigg, 2001). A role for Aurora-A (Katayama et al., 2001; Marumoto et al., 2003) and Aurora-B (Kallio et al., 2002; Murata-Hori and Wang, 2002a) in chromosome alignment has been implicated by antibody microinjection experiments and by ectopic expression of a kinase-inactive mutant. Perturbation of Aurora-B by chemical inhibitors or RNAi further demonstrated a role for this protein in the correction of kinetochore-microtubule misattachment (Hauf et al., 2003). Despite these insights, however, the molecular pathways by which members of the Aurora family regulate kinetochore function have remained unknown.

We now show that Aurora-A, as well as Aurora-B, is important in regulation of kinetochore function in human cells. In a screen for binding partners of Aurora-A, we identified CENP-A. Aurora-A phosphorylated CENP-A in

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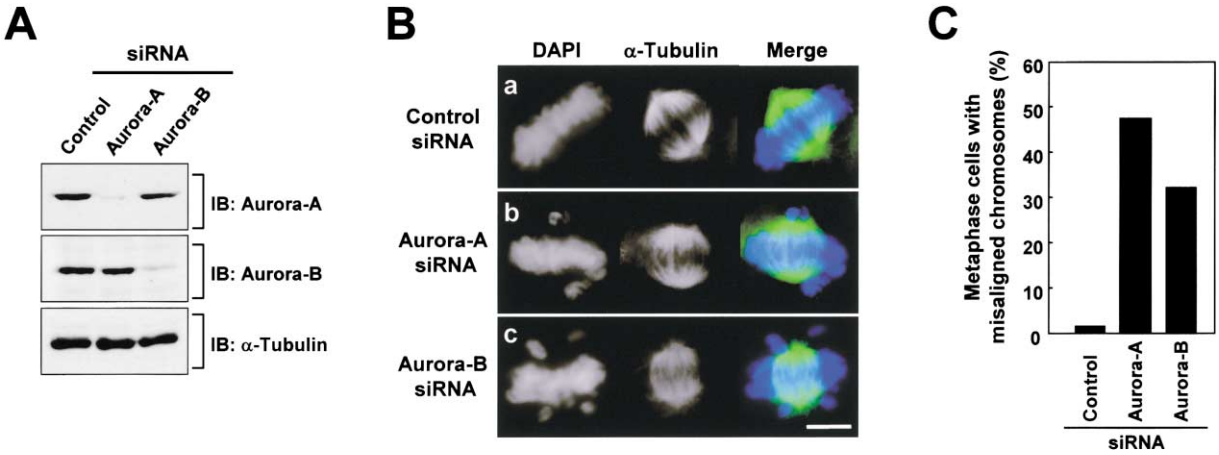

Figure 1. Roles of Aurora-A and Aurora-B in Chromosome Alignment

(A) Depletion of Aurora-A and Aurora-B by RNAi in HeLa cells. Cells were transfected with control, Aurora-A, or Aurora-B siRNAs in the interval between the two thymidine blocks of the double-thymidine block protocol for cell synchronization. Cell lysates were prepared 7.5 hr after release of the cells from the G1-S boundary and were subjected to immunoblot analysis (IB) with antibodies to Aurora-A, Aurora-B, or α-tubulin (control), as indicated.

(B) Defect in chromosome alignment in cells depleted of Aurora-A or Aurora-B. HeLa cells transfected with control (panel a), Aurora-A (panel b), or Aurora-B (panel c) siRNAs as in (A) were subjected to immunofluorescence analysis with antibodies to α-tubulin (FITC, green) and to staining with DAPI (blue) 8.5 hr after release from the G1-S boundary. Representative images of metaphase cells with aligned chromosomes (panels a) or with misaligned chromosomes (panels b and c) are shown. The scale bar represents 10 μm.

(C) Incidence of chromosome misalignment in HeLa cell cultures transfected with control, Aurora-A, or Aurora-B siRNAs. About 100 metaphase cells were scored for chromosome misalignment. Cells in metaphase were identified on the basis of a bipolar spindle configuration and the presence of most condensed chromosomes at the metaphase plate.

vitro on Ser-7, a residue that is also phosphorylated by Aurora-B (Zeitlin et al., 2001b). Examination of the role of both kinases in the phosphorylation of CENP-A revealed that this reaction is mediated sequentially by Aurora-A and Aurora-B in early mitosis. Mitotic cells in which the phosphorylation of CENP-A on Ser-7 was prevented, exhibited a substantial proportion of misaligned chromosomes as a result of a defect in the ability of kinetochores to attach to microtubules. Phosphorylation of CENP-A by Aurora-A in prophase nuclei is thus essential for kinetochore function in mitosis.

Results

Roles of Aurora-A and Aurora-B in Chromosome Alignment at Metaphase

To investigate the roles of Aurora kinases in chromosome alignment in mammalian cells, we first attempted to knock down Aurora-A and Aurora-B by application of RNA interference (RNAi) in synchronized HeLa cells. Cells were transfected with small interfering RNAs (siRNAs) during the interval between two sequential thymidine blocks of the double-thymidine block protocol. Immunoblot analysis performed 7.5 hr after the release of cells from the second thymidine block revealed that expression of the respective siRNAs resulted in a marked depletion of Aurora-A or Aurora-B (Figure 1A).

In cultures transfected with the Aurora-B siRNA, mitotic cells without detectable Aurora-B frequently contained a few chromosomes that failed to align at the metaphase plate (Figures 1B and 1C; Ditchfield et al., 2003; Hauf et al., 2003). As we have recently reported (Hirota et al., 2003), depletion of Aurora-A blocked mitotic entry in HeLa cells. However, the incompleteness of the RNAi experiment allowed us to examine the mitotic cells with reduced levels of Aurora-A; a small fraction (~10%) of cells were observed to enter mitosis after transfection of Aurora-A siRNA, and these cells always showed reduced but still detectable levels of Aurora-A (Supplemental Figure S1A at http://www.developmentalcell.com/cgi/content/full/5/6/853/DC1). As observed with Aurora-B RNAi, the mitotic cells with attenuated Aurora-A expression also revealed a defect in chromosome alignment (Figures 1B and 1C). Although the spindle poles appeared less organized in Aurora-A-deficient cells as previously observed (Kufer et al., 2002), the bipolar spindles themselves appeared unaffected, suggesting that Aurora-A might contribute to the control of chromosome alignment as well as to spindle pole regulation.

Examination of individual cells by immunostaining showed that expression of Aurora-B was not affected by Aurora-A depletion, and vice versa (Supplemental Figure S1A). Moreover, to examine whether one Aurora kinase is required for the activation of the other, we determined the status of histone H3 phosphorylation on Ser-10 as a readout for the kinase activity of Aurora-B (Adams et al., 2001; Ditchfield et al., 2003; Hauf et al., 2003). The extent of histone H3 phosphorylation was greatly reduced in Aurora-B-deficient cells but was not affected in Aurora-A-reduced cells (Supplemental Figure S1B). Conversely, the extent of phosphorylation of Aurora-A on Thr-288, which is indicative of the activation of this kinase (Walter et al., 2000), was similar in Aurora-B-depleted cells and in control cells (Supplemental Figure S1B). These results thus suggested that Aurora-A and Aurora-B are independently regulated, and that not only Aurora-B but also Aurora-A is important for chromosome alignment.

Identification of an Aurora-A-Interacting Protein

To obtain insight into Aurora-A function in mitosis, we subjected a HeLa cell cDNA library to a yeast two-hybrid screen in order to identify proteins that interact with Aurora-A. Because the kinase domains of Aurora family members are highly conserved, we only used the noncatalytic region of amino acids 1–129 of Aurora-A, which is less conserved among Aurora kinases, as a bait to screen for its binding proteins. Of 2.4×10^7 initial transformants, 135 clones were found to confer both the His^+ and $LacZ^+$ phenotypes. These positive clones were subjected to a secondary screening to eliminate false positives and for confirmation of the specific interaction between Aurora-A and the proteins encoded by the library cDNAs within the yeast cells. From ten confirmed positive clones, we isolated two independent cDNAs encoding full-length CENP-A (Figure 2A).

Interaction of CENP-A with Aurora-A

To examine whether CENP-A interacts with Aurora-A in mammalian cells, we coexpressed the two proteins in human embryonic kidney (HEK) 293T cells. The full-length CENP-A and Aurora-A proteins were tagged at their NH_2 termini with green fluorescent protein (GFP) and the Flag epitope, respectively. Immunoprecipitates prepared from the transfected cells with antibodies to Flag were found to contain GFP-CENP-A (Figure 2B). Conversely, Flag-Aurora-A was detected in GFP-CENP-A immunoprecipitates. These results indicated that CENP-A and Aurora-A can associate in human cells.

We sought to delineate the region of CENP-A responsible for the interaction with Aurora-A by incubation of various truncation mutants of CENP-A fused to glutathione S-transferase (GST; Figure 2C) with purified hexahistidine (His_6)-tagged Aurora-A(1–129), the fragment of Aurora-A used as the bait in the two-hybrid screen. Precipitation of the GST-CENP-A fusion proteins with glutathione-agarose beads revealed that His_6-Aurora-A(1–129) associated with CENP-A(31–70) but not with the other CENP-A mutants tested (Figure 2D). This in vitro binding assay thus suggested that the region of CENP-A comprising amino acids 41–60 is required for binding to Aurora-A.

Phosphorylation of CENP-A by Aurora-A

To determine whether CENP-A might be a substrate for Aurora-A, we performed an in vitro kinase assay with recombinant His_6-Aurora-A, either the wild-type protein or a kinase-defective (KD) mutant (containing a mutated ATP binding site), purified from baculovirus-infected Sf9 cells. Incubation of GST-CENP-A with wild-type His_6-Aurora-A, but not with the mutant enzyme, resulted in its phosphorylation (Figure 3A).

To identify the residues of CENP-A phosphorylated by Aurora-A, we examined the ability of Aurora-A to phosphorylate truncation mutants of CENP-A (Figure 2C). Both CENP-A(1–40) and CENP-A(91–140) were phosphorylated by Aurora-A in vitro, whereas CENP-A(31–70) and CENP-A(61–100) were not (Figure 3B). Given that posttranslational modifications of the NH_2-terminal tail domain of core histones play an important role in regulation of chromatin structure and function (Cheung et al., 2000), we focused our attention on the phosphorylation of CENP-A(1–40). Because CENP-A(31–70) was not phosphorylated, the phosphorylation site seemed to be located within the region comprising residues 1–30. We prepared CENP-A(1–14) and CENP-A(13–30) fragments and found that the former, but not the latter, was phosphorylated by Aurora-A (Figure 3D). Among residues 1–14 of CENP-A, Ser-7 is the only phosphorylatable amino acid residue (Figure 3C). To confirm whether this is the residue targeted by Aurora-A, we prepared a CENP-A(1–14) peptide in which Ser-7 is replaced by alanine. As expected, this S7A mutant was not phosphorylated by Aurora-A (Figure 3D).

Effect of the Docking Interaction of CENP-A with Aurora-A on CENP-A Phosphorylation

Our binding and phosphorylation experiments suggested that Aurora-A can associate with CENP-A via the region comprising amino acids 41–60 and can phosphorylate CENP-A on Ser-7. We hypothesized that the physical interaction between the two proteins might increase the efficiency of the enzymatic reaction. To test this idea, we prepared recombinant GST fusion proteins containing either full-length CENP-A or the Δ41–60 mutant, which lacks the binding domain, and then used these proteins as substrates in the kinase assay. We first verified that CENP-A(Δ41–60) does not bind to His_6-tagged Aurora-A (Figure 2E). The rate and extent of phosphorylation of CENP-A(Δ41–60) by Aurora-A were greatly reduced compared with those apparent for phosphorylation of the full-length protein (Figures 3E and 3F). These observations suggest that the direct interaction of CENP-A with Aurora-A promotes the phosphorylation of the former by the latter.

Phosphorylation of CENP-A in Early Mitotic Cells

To study the regulation of the phosphorylation of CENP-A on Ser-7 in cells, we generated rabbit polyclonal antibodies specific for CENP-A phosphorylated on this residue (S7P), as described by Zeitlin et al. (2001a). Immunoblot analysis confirmed that the purified S7P antibodies did not react with nonphosphorylatable recombinant CENP-A of Ser-7 (Figure 4A), and they detected endogenous CENP-A only in mitotic cell lysates (Figure 4B). Immunofluorescence analysis with S7P antibodies revealed two closely spaced spots of immunoreactivity on mitotic chromosomes in HeLa cells, a pattern identical to that apparent for the centromere detected by antiserum to CREST antigen (data not shown). Preincubation of S7P antibodies with the corresponding S7P peptide prevented the staining of centromeres, whereas the nonphosphorylated peptide (S7OH) had no such effect, confirming that the antibodies recognize phosphorylated Ser-7 but not unphosphorylated Ser-7 (data not shown).

Consistent with the previous characterization (Zeitlin et al., 2001a), CENP-A appeared to be phosphorylated only for a short period in early mitosis; phosphorylation of CENP-A on Ser-7 was first detected in cells with condensed chromosomes during prophase, persisted through metaphase, and was no longer apparent in anaphase. Interphase cells showed no reactivity with the S7P antibodies (Figure 4C).

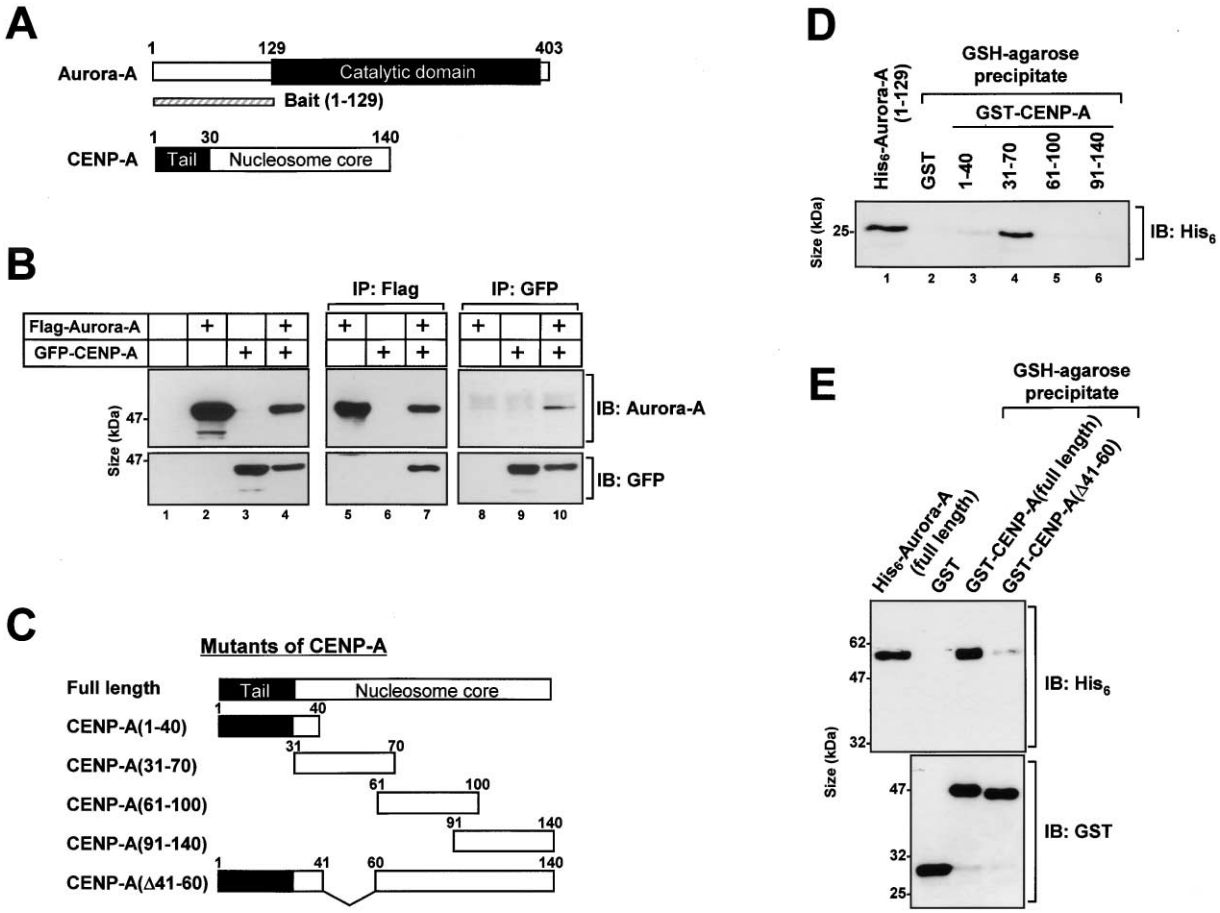

Figure 2. Identification of CENP-A as an Aurora-A Binding Protein

(A) Domain organization of human Aurora-A and human CENP-A. The hatched bar below the Aurora-A structure represents the portion of the protein used as the bait for a two-hybrid screen.

(B) Interaction of CENP-A and Aurora-A in HEK293T cells. Lysates of cells expressing Flag-Aurora-A, GFP-CENP-A, or both proteins were subjected to immunoblot analysis with antibodies to Aurora-A or GFP, as indicated (lanes 1–4). The same cell lysates were also subjected to immunoprecipitation (IP) with anti-Flag (lanes 5–7) or anti-GFP (lanes 8–10), and the resulting precipitates were subjected to immunoblot analysis with antibodies to Aurora-A or to GFP.

(C) Deletion mutants of human CENP-A.

(D) In vitro binding assay with a recombinant fragment of Aurora-A and CENP-A deletion mutants. Purified GST-CENP-A fusion proteins (400 nM), or GST as a control, were incubated with purified His_6-tagged Aurora-A(1–129) (400 nM) and then precipitated with glutathione-agarose beads. Bead-bound proteins were subjected to immunoblot analysis with antibodies to the His_6 tag. The input of His_6-Aurora-A(1–129) into the binding reaction is shown in lane 1.

(E) Requirement for amino acids 41–60 of CENP-A in the binding of CENP-A to Aurora-A. GST-CENP-A(full-length) or GST-CENP-A(Δ41–60) purified from baculovirus-infected Sf9 cells was incubated with purified His_6-tagged full-length Aurora-A and then precipitated with glutathione-agarose beads. Bead-bound proteins were subjected to immunoblot analysis with antibodies to the His_6 tag or to GST.

Accumulation of Aurora-A to the Nucleus in Prophase

Aurora-A has been described as being localized specifically at spindle poles (reviewed in Nigg, 2001). Therefore, we reinvestigated the distribution of this kinase during cell cycle progression by immunofluorescence analysis in order to explore when and where Aurora-A could interact with CENP-A in mitotic cells. Aurora-A became detectable in HeLa cells at G2 phase, exhibiting a diffuse distribution in the cytoplasm, as well as centrosomal enrichment. However, when cells entered prophase, a faint nuclear staining for Aurora-A appeared in cells with condensing chromosomes. The nuclear localization of Aurora-A was most prominent in the later stages of prophase, before nuclear envelope breakdown (Figure 4D, panels a–c). When cells were stained with antibodies specific for activated (Thr-288-phosphorylated) Aurora-A, both centrosomal and nuclear staining was observed (Figure 4D, panel d; Hirota et al., 2003). The active form of Aurora-A therefore appears in the nucleus at the beginning of prophase, concomitant with the initial phosphorylation of CENP-A.

Sequential Phosphorylation of CENP-A by Aurora-A and Aurora-B

Given that Aurora-B had also been previously shown to mediate the phosphorylation of CENP-A on Ser-7 (Zeitlin et al., 2001b), we attempted to distinguish between possible roles of Aurora-A and Aurora-B in the mitotic phosphorylation of CENP-A on this residue. We transfected synchronized HeLa cells with control, Aurora-A, or Aurora-B siRNAs and examined the phosphorylation status

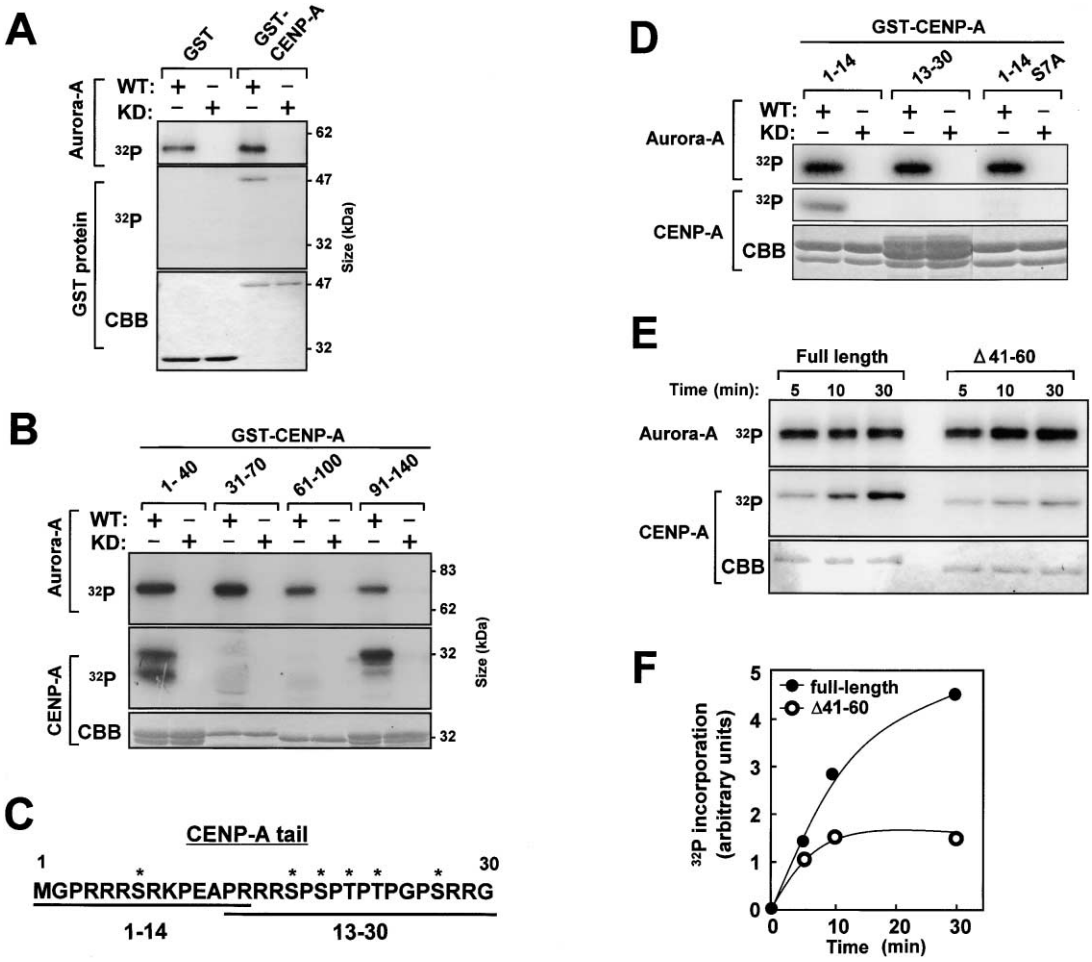

Figure 3. Phosphorylation of CENP-A by Aurora-A In Vitro

(A) Phosphorylation of CENP-A by wild-type but not by kinase-defective Aurora-A. Purified GST-CENP-A or GST was incubated with purified His_6-tagged wild-type (WT) Aurora-A or a kinase-defective (KD) mutant thereof in the presence of [γ-^{32}P]ATP. The kinase reaction mixtures were then analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography (upper and middle panels); the gel was also stained with Coomassie brilliant blue (CBB; lower panel).

(B) Phosphorylation of CENP-A mutants by Aurora-A. Purified GST fusion proteins containing CENP-A deletion mutants (Figure 2C) were incubated with purified GST-Aurora-A in the presence of [γ-^{32}P]ATP. The kinase reaction mixtures were then analyzed as in (A).

(C) Amino acid sequence of the NH_2-terminal tail of human CENP-A. Potential phosphorylation sites are marked by asterisks.

(D) Identification of the NH_2-terminal phosphorylation site of CENP-A targeted by Aurora-A. GST-CENP-A(1–14), GST-CENP-A(13–30), and the S7A mutant of GST-CENP-A(1–14) were assayed for phosphorylation by GST-Aurora-A.

(E) Phosphorylation of a binding-incompetent mutant of CENP-A by Aurora-A. Purified GST-CENP-A(full-length) or GST-CENP-A(Δ41–60) was incubated for the indicated times with purified His_6-tagged wild-type Aurora-A in the presence of [γ-^{32}P]ATP. The reaction mixtures were then analyzed as in (A).

(F) The radioactivity associated with the phosphorylated GST-CENP-A proteins in (E) was quantified by densitometric scanning.

of CENP-A with the S7P antibodies (Figure 5). In the control cells, CENP-A was phosphorylated on Ser-7 from early prophase to metaphase. However, in mitotic cells with a reduced level of Aurora-A, phosphorylation of CENP-A on this residue could not be detected in the majority (~75%) of cells in early prophase and remained undetectable throughout the subsequent mitotic phases. In contrast, in Aurora-B-depleted cells, CENP-A was phosphorylated on Ser-7 in early prophase, but cells positive for S7P staining decreased markedly in number during late prophase and had almost disappeared by prometaphase. These observations suggested that the initial phosphorylation of CENP-A on Ser-7 during prophase is mediated by Aurora-A, but that maintenance of this residue in the phosphorylated state during prometaphase is dependent on Aurora-B and seems to require the prior phosphorylation by Aurora-A. Aurora-A and Aurora-B thus appear to act sequentially to initiate and maintain the phosphorylation of CENP-A on Ser-7.

Nonphosphorylatable CENP-A Induces Aberrant Mitosis

To address the significance of the mitotic phosphorylation of CENP-A on Ser-7, we established HeLa cells that stably express GFP-fused wild-type CENP-A or a nonphosphorylatable mutant (S7A) thereof, in which Ser-7 is replaced by alanine. Both of the GFP-CENP-A proteins specifically localized to the centromere, as

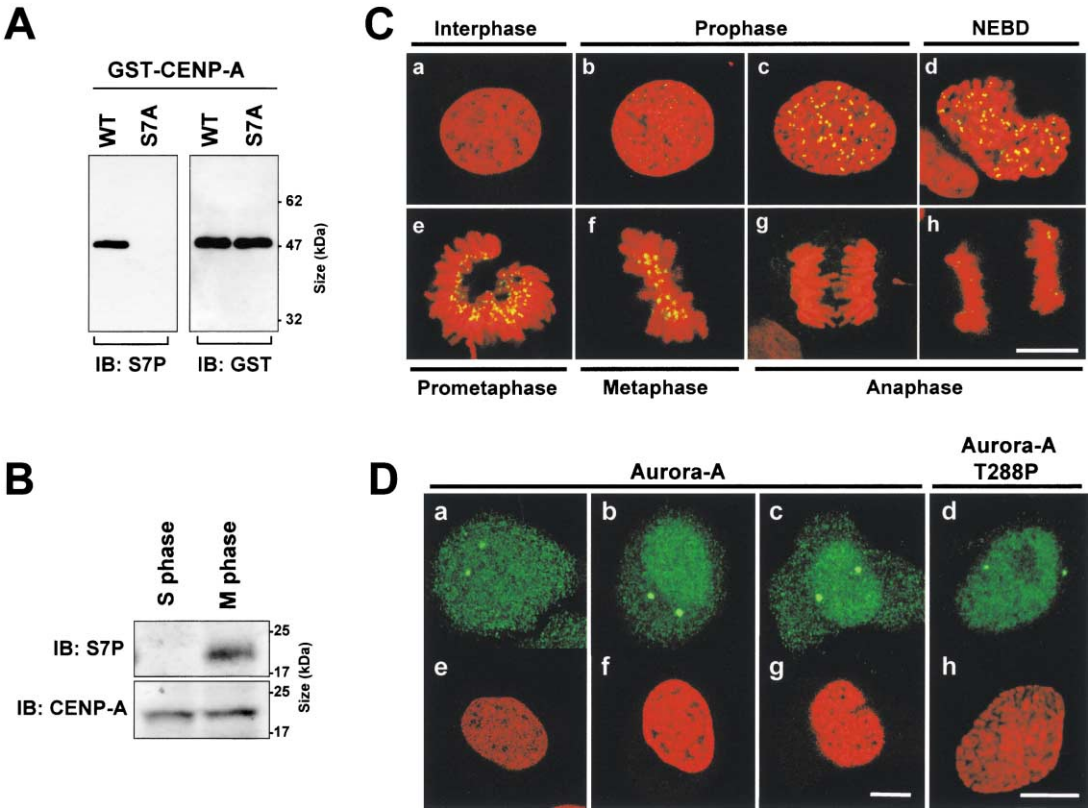

Figure 4. CENP-A Phosphorylation on Ser-7 during Early Mitosis

(A) Specificity of S7P antibodies for CENP-A phosphorylated on Ser-7. GST fusion proteins containing wild-type CENP-A or the S7A mutant were incubated with His_6-tagged Aurora-A in the presence of 10 μM ATP for 30 min. The reaction mixtures were then subjected to immunoblot analysis with S7P antibodies or with anti-GST, as indicated.

(B) Detection of endogenous CENP-A with S7P antibodies. Lysates of HeLa cells in S or M phase were subjected to immunoblot analysis with S7P antibodies or with a mouse monoclonal antibody to CENP-A, which detects both phosphorylated and nonphosphorylated forms of the protein.

(C) Distribution of Ser-7-phosphorylated CENP-A during the cell cycle. Exponentially growing HeLa cells were subjected to staining with propidium iodide (red) and to immunofluorescence analysis with S7P antibodies that had been preincubated with the S7OH peptide (FITC, green). Representative cells in interphase (panel a), early prophase (panel b), late prophase (panel c), during nuclear envelope breakdown (NEBD; panel d), in prometaphase (panel e), metaphase (panel f), and anaphase (panels g and h) are shown. The scale bar represents 10 μm.

(D) Nuclear localization of Aurora-A during prophase. Exponentially growing HeLa cells were subjected to immunofluorescence staining with antibodies to Aurora-A, and representative prophase cells are shown in a temporal order of chromatin condensation (FITC, green; panels a–c) or with antibodies specific for Aurora-A phosphorylated on Thr-288 (FITC, green; panel d). DNA was visualized with propidium iodide (red; panels e–h). The scale bars represent 10 μm.

does endogenous CENP-A (Figure 6A). Analysis of mitotic cells with the S7P antibodies revealed phosphorylation of CENP-A in controls, but such phosphorylation was hardly detectable in cells expressing the S7A mutant (Figure 6A; Supplemental Figure S2B), suggesting that the exogenous expression of this mutant markedly inhibited the phosphorylation of endogenous CENP-A on Ser-7. The CENP-A(S7A) mutant thus appears to act in a dominant-negative manner.

We then monitored these cells at 5 min intervals by time-lapse differential interference contrast (DIC) microscopy. The duration of mitosis was found to vary markedly in cells expressing CENP-A(S7A). Whereas most control cells and cells expressing wild-type CENP-A completed mitosis within 60 min, the majority of cells expressing the mutant protein required more than 60 min (Figure 6B). The prolongation of mitosis seemed to result from a delay in prometaphase (Supplemental Figure S2C).

Immunofluorescence analysis revealed that a substantial proportion of cells in all three GFP-CENP-A(S7A) lines contained chromosomes that failed to align at the metaphase plate (Figures 6C and 6D). Staining of these cells for microtubules revealed monooriented chromosomes that positioned close to poles and appeared to fail to achieve bipolar microtubule attachments, even though bipolar spindles were formed (Figure 6C). To explore the origin of the mitotic delay in cells expressing CENP-A(S7A), we examined the recruitment of checkpoint proteins to kinetochores; such recruitment is thought to occur at unattached, tension-free kinetochores and is involved in the maintenance of checkpoint signaling (Shah and Cleveland, 2000; Hoyt, 2001). We found enrichment of Mad2, BubR1, and CENP-E at the kinetochores of most of the misaligned chromosomes appearing in CENP-A(S7A) mutant cells (Figure 6E). Furthermore, the misaligned chromosomes contained one or two Mad2-positive kinetochores, implicating that these kinetochores were occupied with only few or no microtubules (Figure 6F). These observations suggest that the phosphorylation of CENP-A on Ser-7 during mitosis is required for proper microtubule attachment

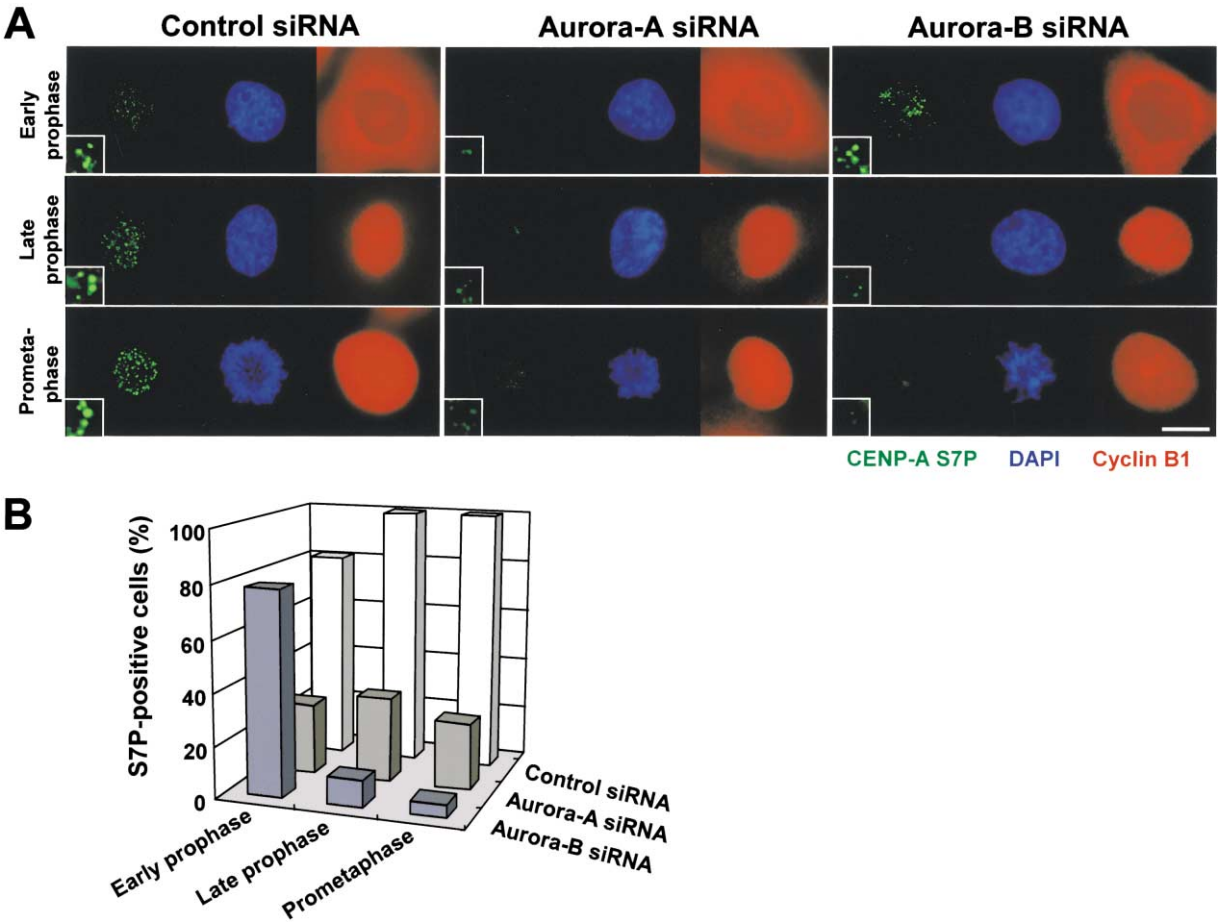

Figure 5. Requirement for Two Aurora Kinases in the Phosphorylation of CENP-A on Ser-7

(A) Immunofluorescence analysis of CENP-A phosphorylation in Aurora-A- or Aurora-B-downregulated cells. Synchronized HeLa cells were transfected with siRNAs as in Figure 1A and subjected to immunofluorescence analysis 8.5 hr after the release from the G1-S block. The cells were stained with S7P antibodies (FITC, green), anti-cyclin B1 (Texas red), and DAPI (blue). They were staged on the basis of chromosome condensation and cyclin B1 localization: cells in early prophase were defined as those with discernible chromosome condensation and marked cytoplasmic accumulation of cyclin B1; cells in late prophase as those with highly condensed chromosomes and nuclear accumulation of cyclin B1; and cells in prometaphase as those with chromatin in the cytoplasm after nuclear envelope breakdown. Representative images of cells in each of these three phases are shown. The scale bar represents 10 μm. Insets present higher-magnification images of S7P staining. (B) Effect of Aurora-A or Aurora-B deficiency on the percentage of cells positive for S7P staining in early mitosis. Cells treated as in (A) were stained with S7P antibodies as well as with either anti-Aurora-A or anti-Aurora-B to exclude those in which RNAi was not effective. Fifty to 100 cells in each of the three phases of the cell cycle examined, classified based on chromatin appearance, were then scored for S7P staining.

at kinetochores, and that the mitotic delay in cells expressing CENP-A(S7A) is attributable to the spindle assembly checkpoint.

Phosphorylation of CENP-A on Ser-7 Is Required for Concentration of Aurora-B at Inner Centromeres

In prophase, the distribution of chromatin-associated Aurora-B becomes restricted to the region between two sister kinetochores called the inner centromere (Zeitlin et al., 2001b; Cleveland et al., 2003). Given that the initial phosphorylation of CENP-A by Aurora-A seems to be necessary for the subsequent phosphorylation by Aurora-B (Figure 5), we hypothesized that the phosphorylation of CENP-A on Ser-7 in prophase might be required for concentration of Aurora-B at the inner centromere. To test this possibility, we examined the localization of Aurora-B in HeLa cells stably expressing GFP-CENP-A(S7A), in which the mitotic phosphorylation of endogenous CENP-A on Ser-7 is impaired (Figure 6A). In all three lines examined, a significant proportion of prometaphase cells failed to exhibit the characteristic accumulation of Aurora-B at the inner centromere; instead, Aurora-B remained associated with the chromosome arms (Figures 7A and 7B).

Measurement of the fluorescence intensity of Aurora-B immunoreactivity in prometaphase cells revealed a substantial signal associated with the chromosome arms in cells expressing CENP-A(S7A) (Figure 7D). However, Aurora-B was also present in the centromere region in these cells, although their intensities were significantly reduced. These observations imply that the abnormal distribution of Aurora-B on prometaphase chromosomes is caused by defects of Aurora-B in dissociating from chromosome arms and localizing efficiently to inner centromeres.

Our RNAi experiments showed that the initial phosphorylation of CENP-A at Ser-7 is dependent on Aurora-A (Figure 5). We therefore compared the effects

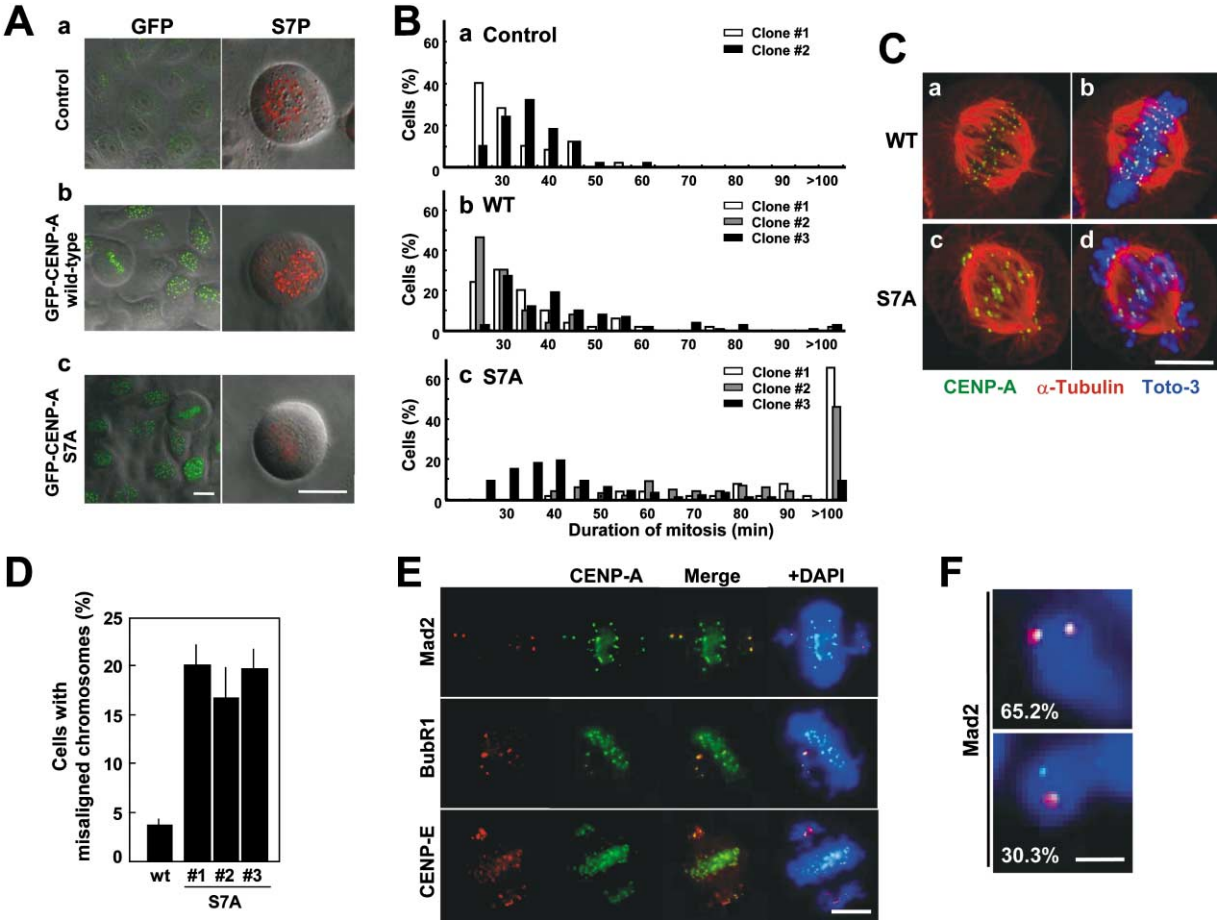

Figure 6. Defective Kinetochore Function in Cells in which CENP-A Phosphorylation Is Blocked

(A) Establishment of HeLa cells stably expressing GFP-CENP-A. HeLa cells were transfected with a plasmid encoding GFP-tagged wild-type CENP-A (panel b) or the mutant CENP-A(S7A) (panel c), or with the corresponding empty vector (panel a). Left panels show GFP fluorescence (green) merged with DIC images. Right panels show indirect immunofluorescence staining with S7P antibodies (rhodamine, red). The scale bars represent 10 μm.

(B) Prolonged mitosis in cells expressing CENP-A(S7A). The duration of mitosis from breakdown of the nuclear envelope (onset of prometaphase) to cytokinesis was measured by time-lapse microscopy. Two, three, and three independent clones of cells stably transfected with empty vector (panel a) or with the vectors for GFP-tagged wild-type CENP-A (panel b) or GFP-CENP-A(S7A) (panel c), respectively, were analyzed. About 100 cells were scored for each experiment. Similar results were obtained with cells that stably express a Myc epitope-tagged CENP-A(S7A) (Supplemental Figure S2D).

(C) Defect in chromosome alignment in cells expressing CENP-A(S7A). HeLa cells stably expressing GFP-tagged wild-type CENP-A (panels a and b) or CENP-A(S7A) (panels c and d) were subjected to immunofluorescence analysis with antibodies to α-tubulin (Texas red). GFP fluorescence is shown in green. DNA was also visualized with Toto-3 iodide (blue; panels b and d). The scale bar represents 10 μm.

(D) Incidence of chromosome misalignment. About 100 metaphase cells expressing GFP-tagged wild-type CENP-A or CENP-A(S7A) were scored for chromosome misalignment. Three independent clones of cells expressing the S7A mutant were analyzed. Data are means ± SD of values from three different experiments.

(E) Association of checkpoint proteins with the kinetochores of misaligned chromosomes. Cells expressing GFP-CENP-A(S7A) were subjected to immunofluorescence analysis with antibodies to Mad2 (upper panels), BubR1 (middle panels), or CENP-E (lower panels) and with rhodamine-labeled secondary antibodies (red). GFP fluorescence is shown in green and DNA was visualized with DAPI (blue). The scale bar represents 10 μm.

(F) Microtubule attachment to kinetochores of misaligned chromosomes. GFP-CENP-A(S7A)-expressing cells that contained misaligned chromosomes (n = 66) were assessed for Mad2 recruitment to kinetochores as in (E); 43 cells (65.2%) contained misaligned chromosomes with Mad2 staining on both kinetochores, and 20 cells (30.3%) had those with Mad2 staining at only one kinetochore, as indicated. Misaligned chromosomes in 3 cells (4.5%) did not show Mad2 staining. The scale bar represents 1 μm.

of Aurora-A reduction to the effects of CENP-A(S7A) expression on mitosis. Synchronized HeLa cells were transfected with Aurora-A siRNA, and cells that entered mitosis were scored for their mitotic duration. The majority of cells with reduced levels of Aurora-A resulted in extension of mitosis (more than 60 min) with prometaphase morphology (Supplemental Figure S2E). They frequently revealed a chromosome alignment defect, as described in Figure 1, and the checkpoint proteins were recruited to kinetochores of those misaligned chromosomes (Supplemental Figure S3). Moreover, in Aurora-A-reduced prometaphase cells, the bulk of Aurora-B remained associated to chromosome arms as we observed in CENP-A(S7A) cells (Figures 7C and 7D). These phenotypic similarities between nonphosphorylatable CENP-A mutants and Aurora-A RNAi cells furthermore

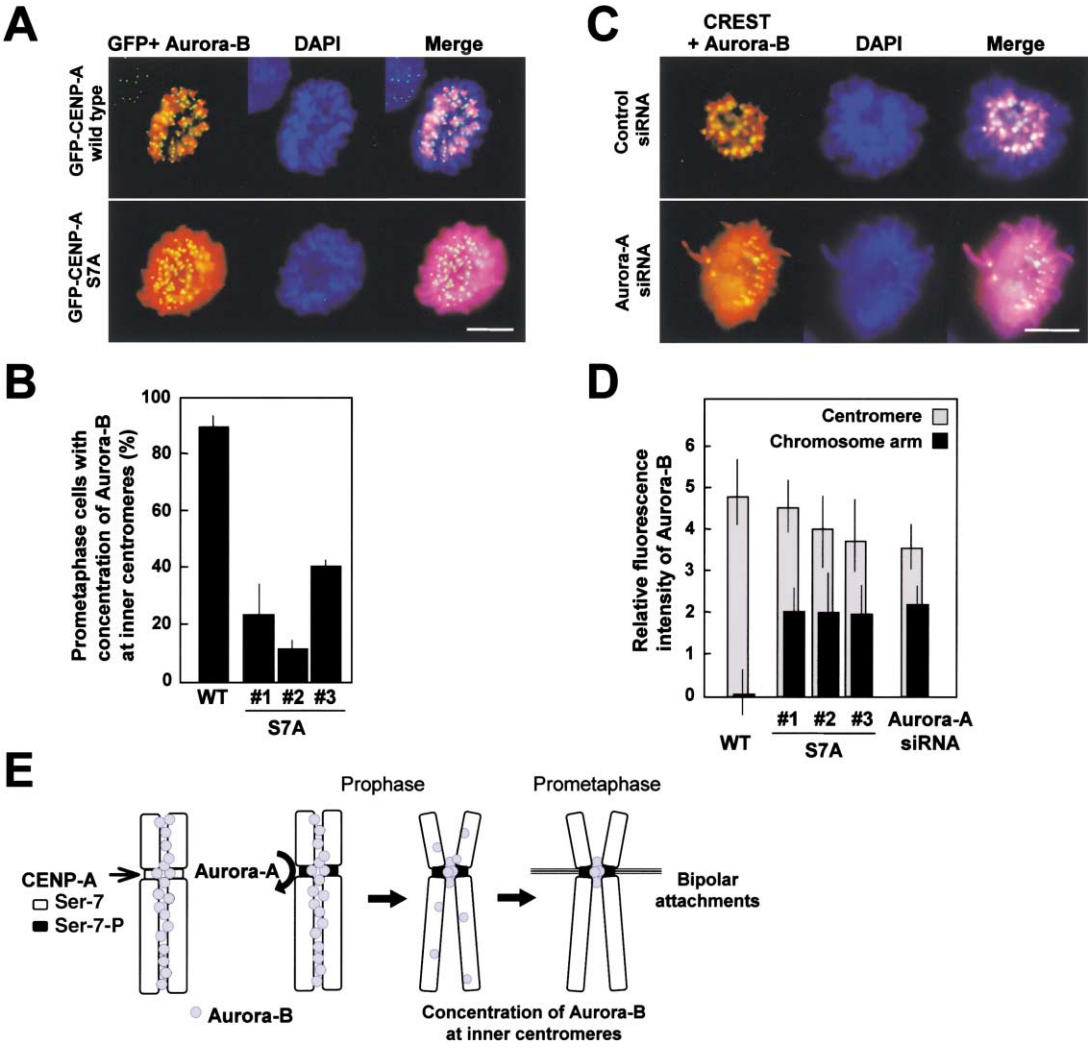

Figure 7. Phosphorylation of CENP-A on Ser-7 Is Required for Concentration of Aurora-B at Inner Centromeres

(A) Failure of Aurora-B to concentrate at inner centromeres in cells expressing CENP-A(S7A). HeLa cells stably expressing GFP-tagged wild-type CENP-A (upper panels) or CENP-A(S7A) (lower panels) were subjected to immunofluorescence analysis with antibodies to Aurora-B (Texas red, red). DNA was visualized with DAPI (blue). Representative images of prometaphase cells are shown. The scale bar represents 10 μm. Similar results were obtained with cells that stably express a Myc-CENP-A(S7A) (data not shown).

(B) Quantitation of cells showing concentration of Aurora-B at the inner centromere. Prometaphase cells (~100) expressing GFP-tagged wild-type CENP-A or CENP-A(S7A) (three different lines) were scored for the concentration of Aurora-B at the inner centromere as detected in (A). Data are means ± SD of values from three independent experiments.

(C) Effect of Aurora-A reduction on Aurora-B distribution in prometaphase cells. HeLa cells transfected with control (upper panels) or Aurora-A (lower panels) siRNAs were subjected to immunofluorescence analysis with antibodies to Aurora-B (Texas red, red) and antiserum to CREST (FITC, green). DNA was visualized with DAPI (blue). The scale bar represents 10 μm.

(D) Fluorescence intensity of Aurora-B immunoreactivity at centromeres and on chromosome arms in prometaphase HeLa cells either expressing GFP-tagged wild-type CENP-A or CENP-A(S7A) or depleted of Aurora-A by RNAi. The centromere was defined as the region between the paired dot-like signals of CENP-A or CREST antigen. The distal portions of chromosome arms were indicated by DAPI staining. All images were collected with the same imaging settings. The intensities of Aurora-B fluorescence were measured and were corrected for background fluorescence. Data are means ± SD of values from 50 cells examined.

(E) Model for the regulation of CENP-A phosphorylation on Ser-7 by Aurora-A and Aurora-B and its role in mitosis.

support that phosphorylation of CENP-A is mediated by Aurora-A.

Discussion

We have shown that the phosphorylation of CENP-A on Ser-7 is important for the proper attachment of microtubules to the kinetochore and consequently for chromosome alignment and segregation. Our results are consistent with a model in which CENP-A is first phosphorylated by Aurora-A during prophase and in which this reaction is required for the restriction of Aurora-B to the inner centromere. Aurora-B then maintains the phosphorylation of CENP-A on Ser-7 from late prophase through metaphase (Figure 7E). Intriguingly, the affinity of Aurora-A for the CENP-A mutant S7E, in which Ser-7 is replaced by glutamate to mimic the Ser-7-phosphorylated form of CENP-A, appeared less than that for the

wild-type protein (Supplemental Figure S4), suggesting that Aurora-A associates with CENP-A only transiently in order to mediate its phosphorylation. In contrast, the affinity of Aurora-B for CENP-A appeared to be unaffected by the phosphorylation status of Ser-7 (Supplemental Figure S4), a property that might allow Aurora-B to maintain Ser-7 phosphorylation on CENP-A for longer periods of time.

Two Aurora Kinases Are Required for CENP-A Phosphorylation on Ser-7

Despite the high sequence homology between Aurora-A and Aurora-B, growing evidence has suggested that the functions and distribution of these two proteins are distinct. Our results suggest an important and unexpected exception, because our observations indicate that CENP-A is not only a substrate for Aurora-B, as previously reported (Zeitlin et al., 2001b), but also for Aurora-A: (1) Aurora-A interacts with CENP-A, CENP-A serves as a substrate for Aurora-A, and the physical association of these two proteins promotes this enzymatic reaction in vitro; (2) the activated form of Aurora-A accumulates in prophase nuclei, when CENP-A first becomes phosphorylated; (3) the phosphorylation of CENP-A in prophase cells is dependent on Aurora-A; and (4) cells depleted of Aurora-A by RNAi and those expressing the nonphosphorylatable CENP-A mutant show phenotypic similarities.

The restricted localization of Aurora-B to the inner centromere and the phosphorylation of CENP-A seem to be intertwined during mitotic progression; the mislocalization of Aurora-B, which is apparent both in cells expressing CENP-A(S7A) and in those with a reduced level of Aurora-A, suggests that the phosphorylation of CENP-A on Ser-7 is required for the concentration of Aurora-B at the inner centromere. Conversely, this concentration of Aurora-B at the inner centromere during prophase appears to be important for Aurora-B to maintain CENP-A phosphorylation after late prophase.

By which mechanism might CENP-A phosphorylation at the centromere affect the association of Aurora-B with chromosome arms? The answer to this question is unknown, but phosphorylation of CENP-A might affect the whole chromosome structure. Given that these events occur in prophase, it is possible that the distribution of Aurora-B depends on chromosome condensation and separation of the chromosome arms as well as on the structure of centromeric chromatin. Alternatively, centromeres containing phospho-CENP-A might somehow modify Aurora-B molecules when they come into contact with centromeres and thereby promote their restriction to the inner centromere. Supporting this turnover model of Aurora-B, photobleaching analysis has shown that centromeric Aurora-B exchanges dynamically with the cytoplasmic pool of this protein (Murata-Hori and Wang, 2002b).

Role of Mitotic Phosphorylation of CENP-A on Ser-7

We observed that the phosphorylation of CENP-A is involved in efficient occupancy of kinetochores with spindle fibers. Concurrent with CENP-A phosphorylation at early prophase, various proteins assemble at the outer domain of the kinetochore (Jablonski et al., 1998; Hoffman et al., 2001). Given that CENP-A is essential for this assembly process in several species (Howman et al., 2000; Blower and Karpen, 2001; Oegema et al., 2001; Goshima et al., 2003), the phosphorylation of CENP-A on Ser-7 might be required to initiate it during prophase, before the kinetochores begin to attach to microtubules. Such protein recruitment triggered by CENP-A phosphorylation might be important for the establishment of kinetochore-microtubule connections. However, this modification does not appear to be necessary for generation of the spindle assembly checkpoint signal, because Mad2, BubR1, and CENP-E localized normally to kinetochores in prometaphase cells expressing CENP-A(S7A) and these cells showed a marked delay in prometaphase.

Cells expressing CENP-A mutants in which Ser-7 is replaced either by alanine or by glutamate have previously been shown to exhibit abnormalities at the final stage of cytokinesis (Zeitlin et al., 2001b), but they did not reveal an obvious role for CENP-A phosphorylation in kinetochore function. An important difference between the two approaches would be that the ectopic expression of CENP-A(S7A) was under conditional control or was constitutive. Zeitlin et al. (2001b) induced expression of CENP-A 24 hr before their analysis, and the ectopically expressed CENP-A protein levels were comparable to that of endogenous CENP-A, whereas the exogenous CENP-A was expressed more than 10-fold higher than endogenous protein in our cell lines (Supplemental Figure 2A). Accordingly, the majority of endogenous CENP-A was possibly replaced by the abundant CENP-A(S7A), and thus the phosphorylation of Ser-7 of CENP-A was virtually undetectable. Notably, the pulse-chase experiments indicate that CENP-A is a long-lived protein and that the abundance of CENP-A decreased by $\sim$50% per generation and remained detectable for 4 days after repression of its synthesis (Shelby et al., 2000). It thus appears likely that a substantial period might be required for the major replacement of endogenous CENP-A by the ectopically expressed protein. Consistently, inhibition of CENP-A expression by siRNA transfection in HeLa cells resulted in the complete disappearance of the protein after 68 hr, but it was still apparent at centromeres 24 and 48 hr after transfection (Goshima et al., 2003). These observations suggest that CENP-A is stable over several cell cycles and that incorporation of mutant CENP-A at centromeres, and the development of a consequent kinetochore defect, might take a substantial period of time.

Aurora Kinases and Kinetochore Function

Given that Aurora-B plays an important role in correcting kinetochore-microtubule attachment in mammalian cells (Hauf et al., 2003), the mislocalization of Aurora-B might contribute to the defect in chromosome alignment in cells expressing CENP-A(S7A) or in those deficient in Aurora-A. However, because Aurora-A-mediated phosphorylation of CENP-A on Ser-7 during prophase appears to be important for microtubule attachment, we were not able to assess the possible contribution of the attachment-correcting function of Aurora-B. We did observe that many misaligned chromosomes in cells in

which CENP-A phosphorylation was prevented possessed either unattached or monotelic kinetochores, whereas those in Aurora-B-depleted cells exhibited syntelic attachment (Hauf et al., 2003; Supplemental Figure S3). Further molecular dissection of the regulation of kinetochore function by Aurora kinases could be facilitated by identification of the proteins that are recruited to the kinetochore in a manner dependent on CENP-A phosphorylation on Ser-7.

Experimental Procedures

Cell Culture and Transfection

HeLa and HEK293T cells were cultured in DME/F12 supplemented with 10% fetal bovine serum and in the absence of antibiotics. HEK293T cells seeded onto six-well plates were subjected to transient transfection with the use of Lipofectamine (Invitrogen). For generation of HeLa cells stably expressing GFP- or Myc epitope-tagged CENP-A, the cells were transfected with the use of the FuGene6 reagent (Roche). Colonies were selected in complete medium containing G418 (800 μg/ml; GIBCO-BRL) and the expanded cells were screened by fluorescence microscopy for uniform ectopic expression of CENP-A displaying characteristic two-paired dot-like signals, either by GFP fluorescence or staining with anti-Myc antibodies. To obtain repetitive results, frozen stocked aliquots of cells were thawed for every experiment. RNAi experiments were conducted as described (Hirota et al., 2003).

Two-Hybrid Screen

The two-hybrid screen was performed as described (Hirota et al., 2003).

Expression Plasmids

Mammalian expression plasmids were constructed by subcloning DNA fragments amplified by the polymerase chain reaction (PCR) into pEGFP-C1 or into pcDNA3 containing the Flag cDNA sequence. All PCR products were obtained with PyroBest DNA polymerase (Takara) and their sequences were verified. For the production of GST fusion proteins and His_6-tagged proteins in bacteria, PCR products were subcloned into pGEX4T-1 (Amersham Pharmacia) and pRSET (Invitrogen), respectively. For protein expression in Sf9 insect cells, DNA fragments were cloned into pFastBac (GIBCO-BRL).

Recombinant Proteins

Escherichia coli strain BL21-S1 harboring either pGEX4T-1-encoding CENP-A mutants or pRSET-Aurora-A was cultured in LB broth for 20 hr at 25°C in the presence of 0.1 mM isopropyl-β-D-thiogalactopyranoside or 0.3 M NaCl. They were then sonicated six times (for 15 s in each sonication) on ice in a solution containing 0.5% NP-40 detergent, 50 mM Tris-HCl (pH 7.5), 25% sucrose, 5 mM $MgCl_2$, 5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, leupeptin (1 μg/ml), pepstatin A (0.7 μg/ml), and aprotinin (5 μg/ml). Soluble proteins were then subjected to chromatography with glutathione-agarose beads (Sigma) or Talon metal affinity resin (Clontech); elution was performed with 5 mM glutathione or 100 mM imidazole, respectively. Recombinant baculoviruses were generated with the Bac-to-Bac baculovirus expression system (GIBCO-BRL). Sf9 cells were infected for 48 hr with baculoviruses encoding His_6-tagged Aurora-A, GST-Aurora-A, or GST-CENP-A (full-length or Δ41–60 mutant); they were then lysed on ice for 10 min in a solution containing 1% CHAPS, 10 mM HEPES-NaOH (pH 7.7), 150 mM NaCl, 100 nM okadaic acid, 20 mM β-glycerophosphate, 5 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, leupeptin (1 μg/ml), pepstatin A (0.7 μg/ml), and aprotinin (5 μg/ml).

Antibodies

Polyclonal antibodies to the phospho-Ser-7 form of CENP-A were generated as described (Zeitlin et al., 2001a). In brief, a synthetic peptide that corresponded to the Ser-7-phosphorylated form of the NH_2-terminal region of human CENP-A (MGPRRRS[PO_3H_2]RKPEAP; S7P) and was coupled to keyhole limpet hemocyanin via an added COOH-terminal cysteine (Peptide Institute, Osaka) was injected into rabbits, and the resulting antisera were subjected to sequential affinity purification on S7OH and S7P peptide columns. Rabbit polyclonal antibodies to Aurora-A were generated as previously described (Marumoto et al., 2002). Polyclonal antibodies to Mad2 were generated in rabbits by injection with recombinant Mad2 protein. Antibodies specific for the phospho-Thr-288 form of Aurora-A and for phosphorylated histone H3 (6G3) were from Cell Signaling Technology; antibodies to α-tubulin (B512) were from Sigma, and those to Aurora-B and cyclin B1 were from Transduction Laboratories. Antibodies to His_6 and GST were from Qiagen and Amersham Pharmacia Biotech, respectively.

Binding and Kinase Assays

These assays were essentially performed as described (Hirota et al., 2003). Anti-Flag (M2; Sigma) coupled to agarose beads or with anti-GFP (3E6; Wako) coupled to protein A-Sepharose beads (Amersham Pharmacia Biotech) were used for the immunoprecipitation. The radioactivity associated with the substrate bands was quantified with a BAS2000 Bioimager (Fujifilm).

Immunofluorescence Microscopy and Image Quantitation

HeLa cells were grown in 35 mm petri dishes, fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) for 20 min at room temperature, and permeabilized with 0.5% Triton X-100 in phosphate-buffered saline. After incubation for 60 min with 5% bovine serum albumin in phosphate-buffered saline, the cells were incubated first at room temperature overnight with primary antibodies and then for 60 min at room temperature with fluorescein isothiocyanate (FITC)-, Texas red-, or rhodamine-conjugated goat secondary antibodies (Biosource or Molecular Probes). Chromatin was labeled with Toto3 iodide (Molecular Probes), 4′,6-diamidino-2-phenylindole (DAPI), or propidium iodide. The stained cells were examined with a confocal microscope (FV300; Olympus) or a fluorescence microscope (BX51; Olympus). Mean fluorescence intensities of antigens at kinetochores or on chromosome arms were measured with a constant circular area and quantitated with Image-J software.

Time-Lapse Microscopy

Cells were grown in 35 mm Delta-T dishes (Bioptechs) in Leibovitz's CO_2-independent medium (GIBCO). Time-lapse series were generated by collecting images every 5 min, and single focal planes were acquired in lieu of *z* sections. Each exposure time was 100 ms. Images were acquired with a Sensys-CCD camera controlled by Metamorph imaging software (Universal Imaging). Cells were maintained at 37°C in a humid chamber, and mineral oil was overlaid on the medium to minimize pH changes and to avoid evaporation.

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